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Reduced glycogen availability is associated with an elevation in HSP72 in contracting human skeletal muscle

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To test the hypothesis that a decrease in intramuscular glycogen availability may stimulate heat shock protein expression, seven men depleted one leg of muscle glycogen the day before performing 4–5 h of exhaustive, two-legged knee extensor exercise at 40 % of leg peak power output. Subjects then rested for a further 3 h. Muscle biopsies were obtained from the depleted and control leg before, immediately after and 3 h into recovery from exercise. These samples were analysed for muscle glycogen, and HSP72 gene and protein expression. In addition, catheters were placed in one femoral artery and both femoral veins and blood was sampled from these catheters prior to exercise and at 1 h intervals during exercise and into recovery for the measurement of arterial–venous differences in serum HSP72. Plasma creatine kinase (CK) was also measured from arterial blood samples. Pre-exercise muscle glycogen content was 40 % lower in the depleted compared with the control leg and this difference was maintained throughout the experiment ($P < 0.05$; main treatment effect). Neither HSP72 gene nor protein expression was different pre-exercise. However, both HSP72 gene and protein increased ($P < 0.05$) post-exercise in the depleted leg, but not in the control leg. Exercise did not increase plasma CK concentrations and we were unable to detect HSP72 in the serum of any samples. These results demonstrate that while acute, concentric exercise is capable of increasing HSP72 in human skeletal muscle, it does so only when glycogen is reduced to relatively low levels. Hence, our data suggest that HSP72 protein expression is related to glycogen availability. In addition, because CK did not increase and we found no evidence of HSP72 in the venous effluent, our data suggest that skeletal muscle is impermeable to HSP72.

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Heat shock proteins (HSPs) are a group of highly conserved proteins present in the cells of all living organisms. Although expressed in low concentrations in the basal state, they are highly inducible by stress (Locke, 1997). It is well known that HSPs are synthesized to restore the function and structure of denatured proteins in a variety of cell types (Sharp *et al.* 1999). For example, HSPs protect hippocampal neurons against focal ischaemia (Sharp *et al.* 1999) and cardiac muscle against ischaemia reperfusion injury (Marber *et al.* 1995) and endotoxaemia (Lau *et al.* 2000). It is also known that HSPs serve as important molecular chaperones importing proteins across organellular membranes (Hood, 2001). Less is known regarding the function of HSP72 within skeletal muscle. However, recent evidence demonstrates that increased expression of HSP72 (the inducible form of the 70 kDa family of HSPs) in rodent skeletal muscle provides protection against oxidative stress (Molka *et al.* 2000) and impaired excitation–contraction coupling processes (Nosek *et al.* 2000). It appears, therefore, that the primary role of HSP72 in skeletal muscle is to restore normal function and/or provide protection from disrupted cell homeostasis.

Physical exercise increases HSP72 in a variety of tissues in several mammalian species (for review see Locke, 1997) and there is emerging evidence that both HSP72 gene (Puntschart *et al.* 1996; Febbraio & Koukoulas, 2000; Walsh *et al.* 2001) and protein (Khassaf *et al.* 2001; Thompson *et al.* 2001) expression are increased in human skeletal muscle following an acute bout of exercise. Since the primary role of HSP72 in skeletal muscle is to restore normal function and/or provide protection from disrupted cell homeostasis, it has commonly been thought that an increase in cell temperature and/or mechanical damage are the likely causes of HSP72 induction during exercise (Locke, 1997), because both of these cellular events disrupt normal function in muscle cells (Febbraio, 2000). It is not clear whether HSP72 expression can

increase during exercise independent of changes in contracting muscle temperature. However, Skidmore *et al.* (1995) exercised rats in a cool environment so that colonic temperatures did not differ from the basal state and observed an elevated HSP72 content in skeletal muscle, suggesting that factors other than temperature can contribute to the accumulation of HSP72. In addition, while HSP72 expression has been observed in skeletal muscle following eccentric exercise that causes mechanical damage to muscle (Punschart *et al.* 1996; Thompson *et al.* 2001; Walsh *et al.* 2001) we (Febbraio & Koukoulas, 2000) and others (Khassaf *et al.* 2001) have demonstrated that HSP72 expression can be increased during or following concentric 'non-damaging' exercise. Hence it is possible that neither elevated temperature nor mechanical damage is solely responsible for stimulating HSP72 production within contracting skeletal muscle. Of note in our recent study (Febbraio & Koukoulas, 2000) was that the increase in HSP72 gene expression only occurred late during exercise when intramuscular glycogen content was reduced to low levels (< 100 mmol (kg dry wt)$^{-1}$). In addition, synthesis of glucose-related proteins (members of the HSP family of proteins) has been reported to increase in cells deprived of glucose (Sciandra & Subjeck, 1983). Taken together, these two studies suggest that a reduced intramuscular carbohydrate availability may provide the stimulus for HSP72 expression during prolonged exercise. No previous studies have examined the effect of intramuscular carbohydrate availability on HSP72 expression within contracting skeletal muscle and, therefore, this was our first aim. We hypothesized that in a glycogen-depleted contracting muscle, the expression of HSP72 would be augmented, when compared with a muscle with a normal pre-exercise glycogen content.

Although studies in rodents have demonstrated that HSP72 protein expression is elevated within hours following exercise (Locke *et al.* 1990; Salo *et al.* 1991; Skidmore *et al.* 1995; Hernando & Manso, 1997), studies in human skeletal muscle have produced equivocal results. Although HSP72 gene expression increases either during (Febbraio & Koukoulas, 2000; Walsh *et al.* 2001) or immediately following (Punschart *et al.* 1996) exercise, HSP72 protein expression is either unaffected (Punschart *et al.* 1996) or increased 48 h (Thompson *et al.* 2001) or 6 days (Khassaf *et al.* 2001) following an acute exercise bout. Further research examining the time-course for gene transcription to protein translation for HSP72 following exercise is, therefore, warranted and this was our second aim. We hypothesized that HSP72 gene and protein expression would occur immediately after prolonged exercise.

Apart from the intracellular role of HSP72 in providing protection against disruption to cellular homeostasis, evidence is emerging that HSPs may have another role in immune defence in that they may be released into the local extracellular environment by stressed cells as a signal to activate the immune response (for review see Moseley, 2000). We have recently demonstrated that exercise results in an approximate 10-fold increase in circulating HSP72 protein (Walsh *et al.* 2001). Whether HSPs are released from skeletal muscle during exercise is not known, but, given their potential extracellular role, such an experiment is warranted and, therefore, the third aim of this study was to examine this phenomenon. We hypothesized that HSP72 would be released from contracting skeletal muscle.

METHODS

Subjects

Seven healthy, physically active, but not specifically trained male subjects of mean age 26 (range 19–33), mean weight 75 kg (range 70–82) and height 1.84 m (range 1.75–1.96) participated in the study. The study was approved by the Ethical Committee of the Copenhagen and Frederiksberg Communities, Denmark, and performed according to the Declaration of Helsinki. Subjects were informed about the possible risks and discomfort involved before their written consent was obtained.

Experimental protocol

Each subject underwent preliminary exercise tests on the two-legged knee extensor model (Blomstrand & Saltin, 1999). After they had become familiar with this, they underwent a maximal exercise test to determine their individual leg peak power output ($W_{max,leg}$). Thereafter, they performed 2.5 h of two-legged knee extensor exercise at 40 % $W_{max,leg}$ in order to become fully accustomed with performing the exercise for prolonged periods.

At 17.00 h on the day before the experimental trial, the subjects reported to the laboratory and performed 60 min of one-legged cycling followed by 60 min of two-arm cranking at a workload that subjects could tolerate for the duration of the exercise period. This protocol was designed to deplete glycogen from the quadriceps of the exercised leg and minimize resynthesis in this leg, by allowing endogenous glucose production to be distributed to the muscles of the upper body for the period between the depletion protocol and the experimental trial. Apart from being permitted to drink water *ad libitum*, the subjects were instructed to abstain from consuming food and drink from 13.00 h on the day of the depletion protocol until the cessation of the experiment. However, each subject was provided with three eggs, six slices of bacon and 500 ml of diet cola to consume on the evening before the experimental trial and two eggs to consume at 7.00 h on the morning of the experimental trial. These meals provided ~3932 kJ (~960 kJ protein, ~70 kJ carbohydrate, ~2907 kJ fat).

Subjects reported to the laboratory at 7.30 h, voided, changed into appropriate exercise attire and remained supine for the next 1.5 h. After 10 min in supine position, the femoral artery and vein from the right leg and the femoral vein of the left leg were cannulated under local anaesthesia (lidocaine, 20 mg ml^{-1}) as previously described (Steensberg *et al.* 2000). The subjects then exercised for 4–5 h (until exhaustion) at the workload used during the familiarization trial. In order to ensure that the same amount of work was performed by each leg, force transducers were fitted to the leg-kicking apparatus and force was monitored 'on line' via a computer recording. Figure 1 illustrates a representative picture

of the strain-gauge profile for one subject for the depleted and a control leg during the knee extensor exercise. The area under the curve for each kick represents the total force made by the muscle contraction during that particular kick.

Muscle sampling and analysis

Muscle biopsies were obtained from the vastus lateralis of both limbs using the percutaneous biopsy technique with suction at rest (Pre-Ex), immediately following exercise (Post-Ex) and at the cessation of the recovery period (3 h Post-Ex). Each sample was divided into three portions before being frozen in liquid nitrogen. One portion was freeze-dried, dissected free of connective tissue and blood and extracted and analysed for glycogen using enzymatic analysis with fluorometric detection (Passoneau & Lauderdale, 1974).

Gene expression

RNA extraction. The second portion was extracted for total RNA essentially as described by Chomczynski & Sacchi (1987). The muscle tissue was homogenized (Polytron, Ultra-Turrax T8, Ika Labortechnik, Staufen, Germany) in 1 ml TriReagent (Molecular Research Center, Cincinnati, OH, USA). Then 50 ml 1-bromo-3-chloropropane was added and the sample mixed thoroughly. After 5 min the samples were centrifuged at 12 000 *g*, 4 °C for 15 min and the RNA in the aqueous phase was precipitated with 1 vol. isopropanol for 5 min. After centrifugation at 12 000 *g*, 4 °C for 8 min the pellet was washed once with 1 ml 75 % ethanol. The dry pellet was resuspended in 20 ml RNase-free water and stored at −20 °C for later use.

Reverse transcription (RT). For each total RNA sample, 0.1 μg was reverse transcribed in a 10 μl reaction containing 1 × TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM each 2′-deoxynucleoside-5′-triphosphate, 2.5 μM random hexamers, 0.4 U μl^{-1} RNase inhibitor and 1.25 × MultiScribe™ reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Control samples were analysed where total RNA samples received all reagents except the reverse transcriptase (rt^-). The reverse transcription reactions were performed using a GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) with conditions at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. Each reaction was then diluted 1/10 in 0.01 M EDTA, pH 8.0, and stored at −80 °C until further use.

Polymerase chain reaction (PCR). We quantified HSP72 mRNA by real-time PCR using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using a multiplex comparative critical threshold (C_T) method. With this method, a C_T value reflects the cycle number at which the DNA amplification is first detected. In each multiplex reaction, both human HSP72 and 18S mRNA were detected in the one tube, where primers were limited for 18S. This was possible because of the different reporter dyes attached to each TaqMan® probe for HSP72 and 18S, both of which fluoresce at different emission wavelength maxima. In preliminary experiments, we demonstrated no effect on C_T values when we compared multiplex with non-multiplex HSP72 reactions, as well as primer-limited multiplex with non-primer-limited 18S single-tube reactions. For the comparative C_T method, a validation experiment was performed where we demonstrated approximately equal efficiencies of both human HSP72 and 18S amplifications, over different initial template concentrations.

To perform real-time PCR, human HSP72 probe and primers were designed using Primer Express™ Version 1.0 (Applied Biosystems, Foster City, CA, USA) from the human heat shock protein (HSP72) gene sequence (GenBank/EMBL accession numbers M11717 and M15432). A 72bp HSP72 fragment was amplified using the forward primer, 5′-ACCAAGCAGACGCAG ATCTTC-3′ and the reverse primer, 5′-GCCCTCGTACACCTG GATCA-3′ (Sigma Genosys, Castle Hill, NSW, Australia). A TaqMan fluorescent probe, 5′-FAM (6-carboxy-fluorescein)-CC TACTCCGACAACCAACCCGGG-3′ TAMRA (6-carboxy-tetramethylrhodamine) (PE Biosystems, Foster City, CA, USA) was included with the primers in each reaction. The TaqMan probe and primers for 18S were supplied in a control reagent kit (PE Biosystems, Foster City, CA, USA).

The PCR reactions were carried out in 25 μl volumes consisting of 1 × Universal PCR Master Mix (including passive reference), 50 nM TaqMan 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, 100 nM TaqMan human HSP72 probe and 900 nM human HSP72 forward and reverse primers. The concentrations of the HSP72 probe and primers were chosen based on pilot analyses where optimal concentrations were determined. The cDNA (5 ng) and rt^- preparations were amplified using the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Comparative C_T calculations for the expression of HSP72 were all relative to the resting sample for each subject. For each sample, 18S C_T values were subtracted from HSP72 C_T values to derive a ΔC_T value. The resting value for each subject was then subtracted from the exercise samples for each subject to derive a $\Delta - \Delta C_T$ value. The expression of human HSP72 relative to the resting sample was then evaluated using the expression $2^{-\Delta-\Delta C_T}$.

Protein expression

An enzyme-linked immunosorbent assay (EIA) method (#EKS-700, StressGen Biotechnologies Corp., Victoria, BC, Canada) was used to determine the relative expression of HSP72 in the third portion of muscle. Briefly, muscle was extracted for protein using a solublizing protein extraction buffer (StressGen Biotechnologies Corp., Victoria, BC, Canada) with the addition of 14 μl (mg tissue)$^{-1}$ of 0.1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF). Samples were homogenized (AG Polytron PT3100 Kinematica, Lucerne, Switzerland) and centrifuged at 20 200 *g* for 10 min at 4 °C according to the instructions provided (StressGen, Biotechnologies Corp., Victoria, BC, Canada) and the supernatants were collected and determined for total protein content (Bradford, 1976). Tissue extracts and known amounts of a protein standard (bovine serum albumin) were incubated with a protein-binding dye (Bio-Rad Laboratories, CA, USA) and absorbance was measured using a microplate reader at 600 nm. The HSP72 was measured with an enzyme-linked immunosorbent assay (StressGen Biotechnologies Corp., Victoria, BC, Canada). Briefly, a mouse monoclonal antibody specific for HSP72 was pre-coated on the wells of the HSP72 immunoassay plate. The HSP72 was captured by the immobilized antibody and was detected with an HSP72-specific, biotinylated rabbit polyclonal antibody. The biotinylated detector antibody was subsequently bound by an avidin–horseradish peroxidase conjugate. The assay was developed with tetramethylbenzidine substrate and stopped with acid stop solution that converted the endpoint colour to yellow. The intensity of the colour was subsequently measured using a microplate reader at 450 nm. The HSP72 concentrations from the samples were quantified by interpolating absorbance readings from a standard curve generated with the calibrated HSP72 protein standard (StressGen Biotechnologies Corp., Victoria, BC, Canada). The traditional method for HSP72 detection and quantification is accomplished in two steps: immunoblotting followed by

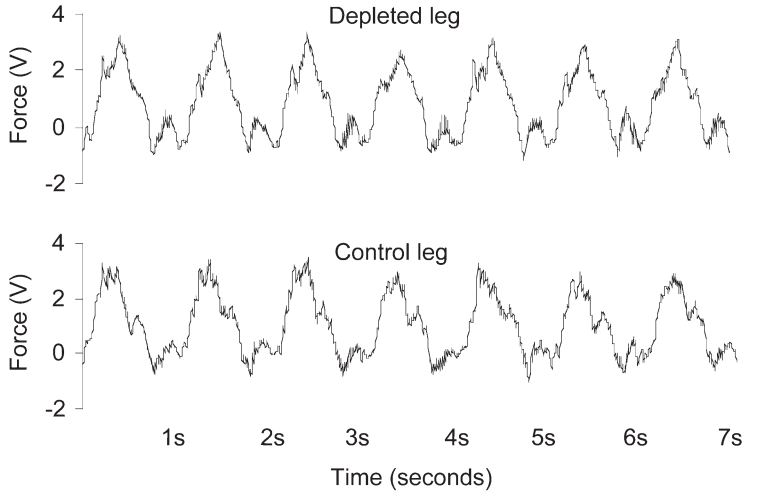

Figure 1

Representative trace of the strain-gauge profile for one subject for a depleted and a control leg during knee extensor exercise

densitometry scanning. We performed an experiment where we quantified the immunosorbent method against this traditional method and demonstrated approximate equal levels of HSP72 in basal muscle samples by both methods (1–4 ng (μg protein)$^{-1}$).

Blood sampling and analysis

Blood samples were collected from the femoral artery and both femoral veins at rest and at 1 h intervals during exercise and into recovery. These samples were allowed to coagulate before being spun and serum collected for later analyses of HSP72 using the immunosorbent assay described above. In addition, at each time point, a blood sample was collected from the artery and these samples, together with a sample collected prior to the depletion exercise protocol, were analysed for creatine kinase (CK) via automated analysis.

Statistics

A two-way (time × treatment) ANOVA with repeated measures was used to compare all data, except for CK, where a one-way ANOVA was performed. Student's Newman–Kuels *post hoc* test was used to locate differences when ANOVA revealed a significant interaction. Significance was set at $P < 0.05$ and all data are reported as means ± S.E.M.

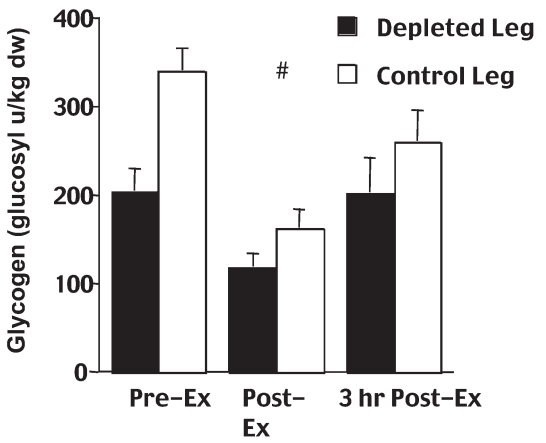

Figure 2. Muscle glycogen content before (Pre-Ex) and following exhaustive knee extensor exercise (Post-Ex) and after 3 h of recovery (3 h Post-Ex) in a glycogen-depleted and control leg

Main effect ($P < 0.05$) for treatment. Data expressed as means ± S.E.M. ($n = 7$).

RESULTS

No differences were observed when comparing the work performed by either limb during the experimental trial (Fig. 1). The exercise bout 16 h before the experimental trial was effective in reducing resting glycogen content by 40 % when comparing the depleted leg (DL) with the control leg (CL) and this difference was maintained throughout the experiment ($P < 0.05$; main treatment effect). Four to five hours of knee extensor exercise reduced ($P < 0.05$) glycogen content in both limbs, but the concentration of this substrate was partially restored after 3 h of recovery (Fig. 2).

Neither HSP72 mRNA nor protein expression was different when comparing DL with CL before exercise (Pre-Ex). However, both HSP72 gene and protein levels increased ($P < 0.05$) approximately two-fold immediately after exercise (Post-Ex) in DL, but not in CL. In DL, HSP72 gene and protein levels 3 h into recovery from exercise (3 h Post-Ex) had returned to that observed Pre-Ex (Fig. 3).

Despite the fact that the detection limit using our immunosorbent assay was as low as 0.8 ng ml^{-1}, and the fact that intramuscular HSP72 concentrations rose to an average of ~200 ng ml^{-1} of homogenized muscle, we were unable to detect any HSP72 protein in the venous or arterial serum at any time point. Plasma CK concentrations were not different when comparing the sample taken before the depletion protocol with the sample taken Pre-Ex. In addition, plasma CK was not elevated at any time point during exercise or recovery when compared with the value observed Pre-Ex (data not shown).

DISCUSSION

The results from this study demonstrate that glycogen availability affects the exercise-induced increase in HSP72 expression in human skeletal muscle, because HSP72 expression only increased in the leg that was glycogen depleted prior to the exercise bout. In addition, since we

could not detect HSP72 in the venous effluent from the glycogen-depleted leg, and there was no sign of cyto-skeletal damage as measured by creatine kinase levels, the data suggest that intact skeletal muscle fibres are impermeable to HSP72.

Acute exercise has been demonstrated to increase HSP72 protein expression in rodent skeletal muscle within 3 h of the cessation of exercise (Locke *et al.* 1990; Salo *et al.* 1991; Hernando & Manso, 1997). The results from the present study are the first to demonstrate a similar effect in humans. We have previously demonstrated that HSP72 gene expression is increased progressively throughout prolonged exercise in humans (Febbraio & Koukoulas, 2000), but we did not measure HSP expression in this previous study. Likewise, Puntschart *et al.* (1996) found an increase in HSP72 mRNA immediately after a 30 min exercise bout, but did not find an increase in HSP72 protein expression within 3 h of exercise. The authors of this previous study speculated that either protein translation was delayed beyond 3 h, or newly synthesized protein was negligible compared with pre-exercising levels. Based on our current results, the latter hypothesis seems plausible, because in the leg depleted of glycogen, both HSP72 gene and protein expression were elevated Post-Ex, but had returned to basal levels 3 h after exercise (Fig. 3). The fact that HSP72 protein expression in the glycogen-depleted leg had returned to basal levels 3 h following exercise was surprising but, nonetheless, consistent with previous observations in rodent skeletal muscle (Locke *et al.* 1990; Salo *et al.* 1991; Hernando & Manso, 1997). Hernando & Manso (1997) suggested that phosphorylation of the protein is the mechanism for generating a specific isoform of HSP72, which is an early event in response of skeletal muscle fibres to exercise stress. This, however, is speculative and further research examining the mechanisms of HSP72 induction during exercise and reduction following exercise is warranted. It must be noted, however, that in contrast with our present data, recent studies have demonstrated that the time course for an exercise-induced increase in HSP72 protein expression in human skeletal muscle is slower. Thompson *et al.* (2001) have recently demonstrated that HSP72 protein expression is increased 48 h following exercise. It must be noted, however, that in contrast with the present study, this previous study used eccentric exercise that caused muscle damage, and it is likely that the stimulus for the HSP72 response was different. Khassaf *et al.* (2001) have recently observed that 45 min of non-damaging exercise does not increase HSP72 protein until 6 days following the exercise bout. In this recent study, HSP72 mRNA measures were not made, but given that the current and three previous (Puntschart *et al.* 1996; Febbraio & Koukoulas, 2000; Walsh *et al.* 2001) studies have consistently demonstrated that HSP72 mRNA rapidly increases in human skeletal muscle in response to exercise, the data from this previous study (Khassaf *et al.* 2001) are somewhat surprising and it must be noted that the authors acknowledged that there was variability in protein measurements. The reason/s why our data appear to appear to contrast this recent study (Khassaf *et al.* 2001) is/are not readily apparent, but clearly more research in the area of exercise-induced HSP72 expression in human skeletal muscle is warranted.

Interestingly, even though the control leg (CL) was subjected to > 4 h of contraction, we were unable to detect any increase in HSP72 gene or protein expression. This was despite the fact that Post-Ex glycogen levels in CL were

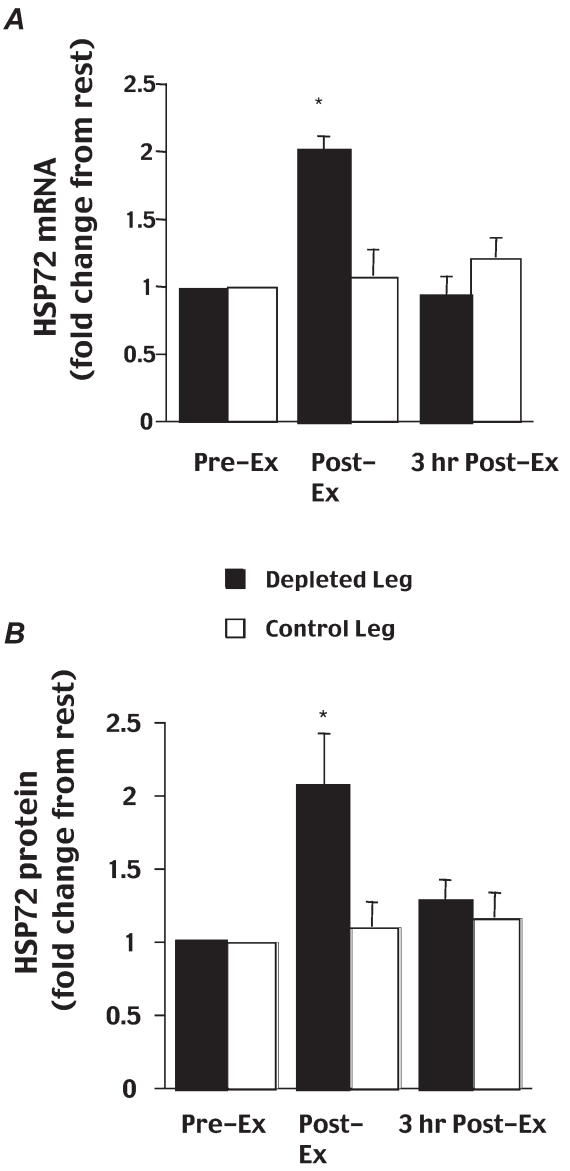

Figure 3. HSP72 mRNA (*A*) and protein (*B*) before (Pre-Ex) and following exhaustive knee extensor exercise (Post-Ex) and after 3 h of recovery (3 h Post-Ex) in a glycogen-depleted and control leg

* Difference ($P < 0.05$) when comparing depleted with control leg. Data expressed as means ± S.E.M. ($n = 7$).

approximately equal to Pre-Ex glycogen levels in the depleted leg (DL; Fig. 2). Nonetheless, after exercise glycogen content was still lower in DL compared with CL. It is possible, therefore, that a threshold exists whereby glycogen must reach a critically low level before HSP72 gene and subsequent protein expression is increased. HSP72 gene and protein expression Pre-Ex were not different when comparing limbs, and this suggests that the exercise the preceding day was not responsible for the elevated HSP72 Post-Ex. We cannot, however, rule out the possibility that the preceding exercise resulted in increasing HSP72 expression and acknowledge it as a limitation to the study.

We have previously demonstrated that the protocol used in this study does not result in differences in blood perfusion when comparing the depleted with the control leg (Blomstrand & Saltin, 1999) making the possibility that oxygen availability or pH were different when comparing limbs most unlikely. Therefore, we cannot attribute the difference in HSP72 protein expression when comparing limbs to any chemical damage caused by altered pH or reduced oxygen availability. Likewise, although we did not measure muscle temperature in this study, differences in this measure when comparing limbs is unlikely. Since the amount of work completed, and hence the amount of heat generated, was the same when comparing limbs, heat production and heat dissipation was likely to be equal when comparing limbs. We can, therefore, exclude the possibility that the exercise-induced expression of HSP72 when comparing DL with CL was due to any differences in intramuscular temperature. Finally, the two-legged knee extensor exercise model adopted in this study is purely concentric in nature and unlikely to have caused any mechanical damage to the muscles, as evidenced by our inability to detect changes in plasma CK. However, even if a small amount of mechanical damage occurred, it would probably have occurred in both limbs since both limbs did the same amount of work (Fig. 1). Therefore, we can rule out mechanical damage as the mechanism for inducing HSP72 protein expression in the current study. Hence, these data do not support the notion that cellular damage and/or temperature are the only mechanisms for contraction-induced increases in HSP72 protein expression in skeletal muscle.

Although the synthesis of glucose-related proteins (members of the HSP family of proteins) has been reported to increase in cells deprived of glucose (Sciandra & Subjeck, 1983), this is the first *in vivo* study linking HSP72 synthesis to carbohydrate availability. There are several factors that may cause glycogen availability *per se* to increase HSP72 protein synthesis. The HSP72 protein has a two-domain structure, with the N-terminal end binding to adenine nucleotides, and the C-terminal end binding to polypeptides (Hightower *et al.* 1994). In order to increase HSP72 gene transcription, the heat shock transcription factor (HSF) must be activated. When ATP content is reduced in cultured myogenic cells, HSF is found to be activated (Benjamin *et al.* 1992). In addition, it has been demonstrated that HSP72 requires ADP as a cofactor for peptide binding, and has a low binding affinity in the presence of ATP (Glick, 1995). Therefore, a change in the ADP/ATP ratio may increase polypeptide-bound HSP72, thereby decreasing the free pool of HSP72 leading to an increase in HSP72 production (Moseley, 2000). We (Baldwin *et al.* 1999) and others (Sahlin *et al.* 1990; Spencer *et al.* 1991) have previously observed that glycogen depletion during exercise results in such a change in the ATP/ADP ratio. It is important to note that HSP72 polypeptide binding during muscle contraction may be a critically important physiological response. Most mitochondrial proteins are derived from nuclear DNA and evidence is emerging that cytosolic HSP72 is a critical protein for chaperoning proteins from the cytosol into the mitochondria (Hood, 2001). Although speculative, it is possible that since the mitochondrion is critical for preserving energy turnover during contraction, a major role for HSP72 during contraction may be to assist in the maintenance of mitochondrial function. It is possible, however, that low carbohydrate availability within muscle cells may lead to other changes within the cell, such as an increase in reactive oxygen species (Reid, 2001) and/or a decrease in sarcoplasmic reticulum calcium re-uptake (Stephenson *et al.* 1999), which are known to activate HSF leading to increased HSP72 transcription.

In the present study, we were unable to detect HSP72 in the arterial serum or the venous serum of each limb, despite a two-fold increase in HSP72 protein expression in DL. This was surprising given that our recent data shows that 1 h of exercise increased the appearance of HSP72 in the forearm venous blood of humans (Walsh *et al.* 2001). It must be noted that, in this previous study, running was used as a mode of exercise. Running largely involves eccentric contractions, which can cause muscle damage, and in this previous study CK was elevated post-exercise. In contrast, in the present study we used a model of exercise that involved purely concentric contractions and CK was not increased during or after exercise. Taken together, these two studies may indicate that intact muscle membranes are not permeable to HSP72, and this protein is only released when the muscle membrane is damaged. Further investigation into the release of HSP72 into the extracellular environment and the biological role of such a release is warranted.

In summary, this study demonstrates that HSP72 increases in contracting skeletal muscle after acute exercise, but only in a muscle that has been previously depleted of glycogen by prior exercise. In addition, it appears that HSP72 is not released into the circulation during or after prolonged concentric exercise.

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